

ORIGINAL ARTICLE

Conditions associated with very low values of glycohaemoglobin measured by an HPLC method

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Aims: To identify the causes of very low glycohaemoglobin (GHb) values in a sample of patients with diabetes in southern Brazil using high performance liquid chromatography.

Methods: Between August 1996 and December 2001 all samples from patients with diabetes at a university hospital with GHb values below the reference range (4.7–6.0% HbA_{1c}) were submitted to cellulose acetate electrophoresis. Medical records were reviewed to identify conditions that might be associated with these low values.

Results: Among 29 657 samples analysed, 130 patients had GHb < 4.7%. Seventy three patients (56%) were heterozygous for HbS, HbC, or HbD (19 black, two mulatto, and 52 white patients). The other 57 patients (44%) without Hb variants had low haematocrit and haemoglobin values (42 patients) or other conditions such as pregnancy, lipaemia, malignancy, cirrhosis, acetylsalicylic acid use, and absence of diabetes (15 patients).

Conclusions: The presence of an Hb variant may falsely lower GHb measurements. However, anaemia is also a source of negative interference. The haematological status should be considered for the correct interpretation of GHb results.

The glycohaemoglobin (GHb) test is an invaluable tool for monitoring blood glucose control over time, and as such it is a key issue in diabetes care and management. According to the American Diabetes Association, GHb should be measured regularly in all patients with diabetes, and values should be maintained below 7% to prevent and/or decrease the risk of chronic complications.¹

GHb is formed in vivo by a reaction between glucose and the N-terminal region of haemoglobin (Hb) α or β chains.² This irreversible non-enzymatic reaction between glucose and haemoglobin A, the main type of Hb in normal adults, occurs over the life span of the erythrocyte. The resulting HbA_{1c} (glycated haemoglobin) is a stable GHb containing primarily glycated N-terminal chains,³ and its total amount depends directly on the average glucose concentration over the two to three months before the measurement.^{4–5}

“The prevalence of the most common haemoglobin variants (HbS, HbC, and HbD) depends on the genetic background of the population being analysed”

However, the presence of Hb variants may falsely lower GHb values. Bry *et al* stressed that the identification of Hb variants is important to avoid inaccurate GHb results.⁶ The prevalence of the most common Hb variants (HbS, HbC, and HbD) depends on the genetic background of the population being analysed. Although relatively rare in white individuals, these variants are common in populations with heterogeneous ethnic backgrounds.⁷ In such populations, misleadingly low GHb values have been identified by some methods, but not by others. The influence of Hb variants on GHb determination has been shown to be method dependent, and also to be greater when ion exchange high performance liquid chromatography (HPLC) is used.^{8–13} In addition, several other factors besides the presence of genetic variants or chemically modified derivatives of haemoglobin,¹³ such as drugs, anaemia, uraemia, and alcoholism, may falsely lower GHb results. Nevertheless, most of these data originate from

method evaluation or validation protocols studies. The magnitude and impact of the effects of these interferences in routine clinical practice have not yet been well established.

Therefore, we analysed the possible causes of very low GHb results measured by HPLC in a sample of patients with diabetes in southern Brazil.

MATERIALS AND METHODS

Samples

From August 1996 to December 2001, 29 657 samples from patients with diabetes attending the outpatient diabetic clinic at Hospital de Clínicas de Porto Alegre, Brazil, were collected for GHb determination. According to the last census,¹⁴ 86% of the state population are classified as white, 8.4% as mulatto, 4.0% as black, and 0.9% as native, yellow, or not defined (classification based on self reporting). The patient population at this centre reflects this ethnic distribution.

Based on the clinical observation of discordant results between GHb measurements and home glucose monitoring in some patients, we decided to submit all samples with GHb values below the lower limit of our reference range (4.7–6.0% HbA_{1c}) to Hb electrophoresis according to the protocol shown in fig 1. Blood was collected in evacuated test tubes containing EDTA. Samples were pretreated to eliminate the labile fraction and stored at 4°C. The analysis was carried out within five days of collection.¹⁵ All medical records were reviewed to identify other conditions or factors that might be associated with low GHb values. Ethnicity was defined by patient self reporting registered in the medical records.

Ten samples from known non-diabetic individuals—five homozygous, and five heterozygous for HbS—as identified by the haematology department and confirmed by Hb electrophoresis, were also analysed for GHb. These samples were

Abbreviations: CV, coefficient of variation; DCCT, Diabetes Control and Complications Trial; GHb, glycohaemoglobin; Hb, haemoglobin; HbA_{1c}, glycated haemoglobin; HPLC, high performance liquid chromatography

Table 1 Characteristics of patients and haemoglobin, haematocrit, and HbA_{1c} results in patients with diabetes with very low values of glycohaemoglobin in southern Brazil

	Hb variant present	Hb variant absent
N	73	57
Sex (male/female)	16/57	32/25
Age range (years)	11–82 (55)	12–66 (41)
Ethnicity	19 blacks, 3 mulatto, 51 whites	4 blacks, 53 whites
Haematocrit*		
vol/vol	0.30–0.50 (0.40)	0.10–0.52 (0.38)
%	30.1–50.3 (40.6)	10.0–51.8 (37.8)
Haemoglobin* (g/l)	95–167 (132)	31–169 (119)
HbA _{1c} * (%)	2.94–4.70 (4.09)	3.40–4.70 (4.43)

Values are range (median).

**p* < 0.001.HbA_{1c}, glycated haemoglobin.

used as controls to test the ability of the GHb method to recognise and measure Hb variants.

Analytical methods

GHb was measured by HPLC (Merck-Hitachi L-9100 Glycated Haemoglobin Analyser; Tokyo, Japan) using a CCMpack Hb-S column in high speed mode. This cation exchange column allows separation of HbA_{1a}, HbA_{1b}, HbF, labile HbA_{1c}, stable HbA_{1c}, and HbA₀. The reference range for GHb (4.7–6.0% HbA_{1c}) was established based on 57 samples from normal individuals (44 women, 13 men, oral glucose tolerance test, World Health Organisation criteria). The laboratory at Hospital de Clínicas de Porto Alegre is regularly monitored by the National Glycohaemoglobin Standardisation Programme to ensure traceability to the Diabetes Control and Complications Trial (DCCT) GHb reference.^{16 17}

Intra-assay and interassay precision were evaluated by measuring samples with different HbA_{1c} values in the same run or in different runs over 20 days. To study the effect of the haematocrit on the assay, whole blood samples (*n* = 5) were spiked with different amounts of their own plasma to obtain haematocrit values ranging from 0.10 to 0.45 vol/vol (10–45%); these blood samples were then analysed for GHb.

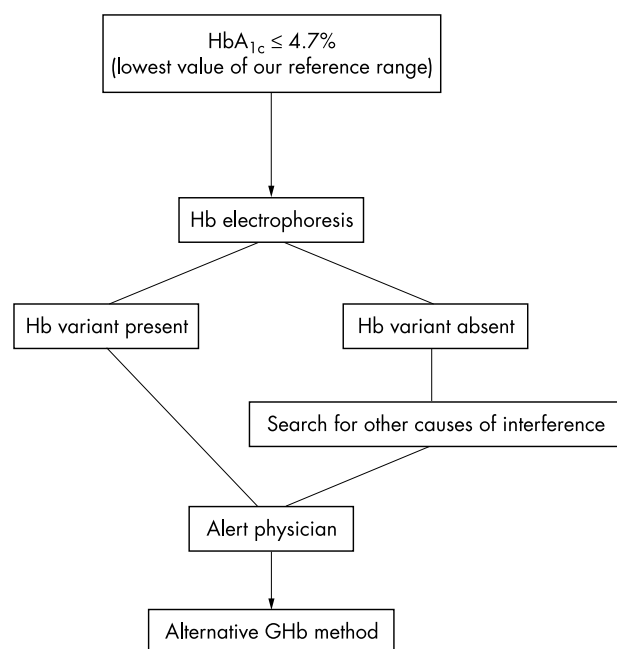


Figure 1 Flow diagram of research protocol used to identify conditions associated with very low glycohaemoglobin (GHb) values.

Hb electrophoresis was performed with cellulose acetate film and Tris/EDTA/borate 0.025M buffer, pH 8.6 (Cellogel, Milan, Italy), and quantification was carried out by elution and reading at 413 nm in a spectrophotometer (Hitachi 2000U; Tokyo, Japan). Samples presenting Hb variants were submitted to qualitative electrophoresis on agar gel with phosphate buffer, pH 6.5 (Bacto-Agar, Kansas City, Missouri, USA), for identification of the Hb type.

Haemograms were performed on a Pentra 2000 Automated System (ABX Diagnostic System, Montpellier, France).

Statistical analysis

The non-parametric Mann Whitney test was used for comparison between groups with a significance level of 5%.

Ethical aspects

The study protocol was approved by the ethics committee of the Hospital de Clínicas de Porto Alegre.

RESULTS

The intra-assay coefficient of variation (CV) was < 1.5% (1.05%, 146%, and 1.37% for low, medium, and high HbA_{1c}

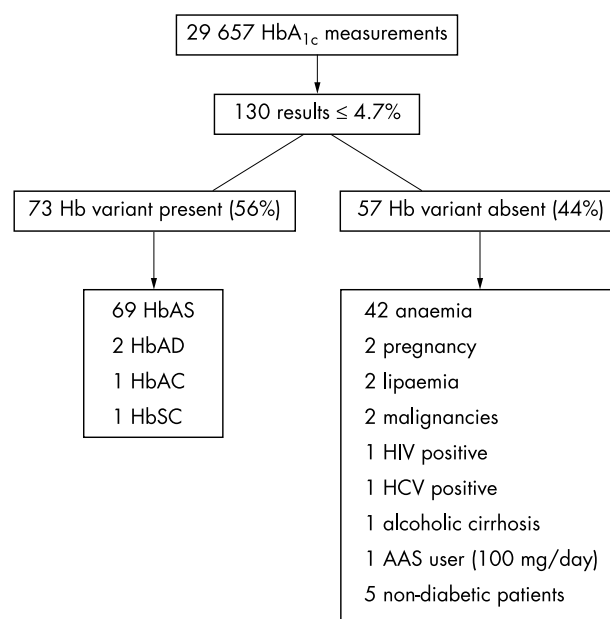


Figure 2 Summary of conditions associated with very low glycohaemoglobin (GHb) values in a group of patients with diabetes in southern Brazil. AAS, acetylsalicylic acid; HbA_{1c}, glycated haemoglobin; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

values, respectively) and the interassay CV was $< 3.0\%$ (2.79%, 2.49%, 1.46% for low, medium, and high HbA_{1c} values, respectively). The haematocrit value did not affect assay precision because there was no difference in GHb results in the same sample with haematocrit values ranging from 0.10 to 0.45 vol/vol (10–45%) *in vitro*.

In total, 29 657 samples were analysed for GHb. Very low GHb values were detected in 130 patients (table 1; fig 2). In 73 patients, an anomalous Hb was identified by electrophoresis: 19 patients were black, three were mulatto, and the others were white.

In the five non-diabetic patients from the haematology division who were homozygous for HbS, HbA_{1c} values were 0%. Their chromatograms showed a double HbA₀ peak that could not be measured by the analyser. The five non-diabetic patients who were heterozygous for HbS had very low GHb values, similar to the patients with diabetes who had HbAS, HbAC, or HbAD. There were no abnormalities in the chromatograms of these patients; there were no extra peaks and no cases of incomplete separation. In the one non-diabetic patient who was heterozygous for HbS and HbC, a double HbA₀ peak that was not quantified by the analyser was also observed.

Except for very low GHb values, the patients with diabetes who were heterozygous for the Hb variants showed no clinical evidence to suggest the presence of a variant.

In 57 (44%) patients with GHb values below 4.7%, no Hb variant was identified. Similar to the 73 patients in whom the Hb variant was identified, these patients showed no alteration in the HPLC chromatograms. However, 42 had low haematocrit values (range, 0.10–0.38; median, 33 vol/vol; range, 10–37.8%; median, 33%; reference range for men, 0.41–0.53 vol/vol and 41–53%; reference range for women, 0.36–0.46 vol/vol and 36–46%), and haemoglobin (range, 31–127 g/litre; median, 107; reference range for men, 135–175 g/litre; reference range for women, 120–160 g/litre). The other 15 patients presented other clinical conditions but no anaemia (fig 2). Two patients had gross lipaemia (triglycerides > 15.8 mmol/litre (6 g/litre). One human immunodeficiency virus positive patient was receiving treatment with antiretroviral agents, itraconazole, sulfadiazin, pirimetamin, and folic acid.

There was a significant difference between the GHb, haematocrit, and Hb values in patients with and without the Hb variants ($p < 0.001$; table 1).

DISCUSSION

Our present study shows that the presence of anomalous Hb is a major cause of very low values of GHb as detected by HPLC. Among our patients presenting GHb values below 4.7%, 56% (70% of whom were classified as white) of the results may be accounted for by the presence of the Hb variant.

These findings are not surprising if one takes into account the ethnic heterogeneity of the Brazilian population. In Brazil, the HbAS genotype is present in about 1% of those persons identified as white. Although according to the last national census 86% of the Brazilian population is white, it is expected that a large number of people carry the HbS gene, confirming the mixed genetic background of this population.^{7 14}

In cases with Hb variants, it has been suggested^{18 19} that “true” HbA_{1c} results may be obtained after appropriate correction based on the peak area for each glycated and non-glycated component separated in the chromatograms. In our case, the HPLC does not recognise Hb variants and the calculation for the glycated component is only related to HbA_{1c}, not to HbS_{1c}, HbC_{1c}, or to HbD_{1c}, resulting in very low GHb values. The mean amount of Hb variants in our samples

was 33% (range, 21.7–44.1%). Therefore, we can assume that the GHb values for these patients who are heterozygous for Hb variants are around 33% higher. It is worth mentioning that some rare variants may have different glycation rates compared with normal HbA.¹⁸ We recommend that laboratories measure GHb by a method that is not affected by Hb variants, rather than estimate it.

Surprisingly, 44% (57 patients) of the very low GHb values we observed could not be accounted for by the presence of an Hb variant. All medical records were reviewed to identify a possible cause for these low GHb values. Five patients had GHb measurements performed during the diagnosis of diabetes (World Health Organisation criteria), with negative results, and two were pregnant. Malignancies, human immunodeficiency virus or hepatitis C virus infection, chronic use of aspirin, lipaemia, and alcoholic cirrhosis were present in eight patients. Apart from alcoholism, lipaemia, and chronic ingestion of salicylates, which affect some GHb assays,¹³ the other conditions have not been reported to interfere with GHb measurements. We believe that the concomitant use of many drugs to treat these patients may have had a GHb lowering effect. Other causes for the negative interference in these cases remain to be elucidated.

“We recommend that laboratories measure glycohaemoglobin by a method that is not affected by haemoglobin variants, rather than estimate it”

Nonetheless, all the conditions seen in our patients without Hb variant explain only 26.3% of these very low GHb results. Most patients who did not have anomalous Hb (73.7%) presented low haematocrit and haemoglobin values for their sex and age, suggesting that anaemia could be associated with lower GHb values. Decreased red blood cell survival and mean erythrocyte age falsely lower GHb values.¹³ Blood loss, haemolytic anaemia, sickle cell anaemia, and chronic renal disease affect the life span of red blood cells and are known to be associated with underestimated GHb values.^{6 20} In contrast, iron and B₁₂ vitamin deficiency have been reported to overestimate GHb results.^{21 22} Our results show that different degrees of anaemia are associated with very low values of GHb. This may be because of small alterations in red blood cell survival or the rate of GHb formation, leading to an underestimation of GHb results. Such alterations cannot be attributed to assay interference, because GHb measurements were precise (CV $< 5\%$) in a

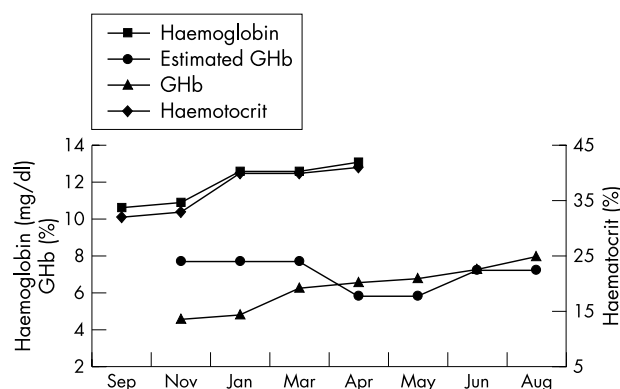


Figure 3 Glycohaemoglobin (GHb), haemoglobin (Hb), and haematocrit (Ht) values in a patient with very low GHb values. The estimated value for GHb, calculated by mean plasma glucose in the period, is shown for three month intervals. After the correction of anaemia, GHb values increased, reaching the estimated value.

Take home messages

- The presence of a haemoglobin variant may falsely lower glycohaemoglobin (GHb) measurements by high performance liquid chromatography
- Anaemia is also a source of negative interference and the haematological status of patients should be considered for the correct interpretation of GHb results

same sample with a haematocrit ranging from 0.10 to 0.45 vol/vol (10–45%) in vitro. Furthermore, intra-assay and interassay precision were acceptable for very low values of GHb (CV < 3%, mean 4.2% HbA_{1c}). Figure 3 shows an example of a patient with minor anaemia and very low GHb. Correction of anaemia was followed by an increase in GHb. The expected value, based on fasting plasma glucose during the period analysed, closely matched the value measured after the correction of the haematocrit and Hb values. However, the DCCT showed that fasting plasma glucose underestimates GHb results, so that the true GHb values for our patient with minor anaemia may be higher.²³

Anaemia seems to have its effects in vivo, probably as a result of reduced red blood cell survival in these patients. If this is true, this in vivo effect will also affect GHb measurements by other methods and assays.

One possible limitation of our study was that it only picked up patients with diabetes whose GHb values were below the lower limit of the reference range. However, some patients with diabetes may have Hb variants, low haematocrit values, or other ill defined interferences and GHb values that do not agree with their home blood glucose monitoring results, even though they are not below the lower limit. The interference may be more clinically relevant, with poor metabolic control. In view of the fact that GHb measurement is a key issue in diabetes care and management, the identification of these patients is very important to monitor their blood glucose control over time. The laboratory should ensure that their GHb results will not be misinterpreted. Physicians must be aware of the possibility of such interference in the clinical setting and should contact laboratories if discrepancies between clinical impressions and laboratory data are seen. Another limitation was the HPLC system used. Although it belongs to the first generation of dedicated HbA_{1c} HPLC systems, it is DCCT traceable and very precise. Modern HPLC systems are now able to identify and quantify Hb variants precisely and accurately. However, we believe that the experience reported here may be useful for other laboratories, using different methods, to guarantee the results of their GHb measurements by controlling possible pitfalls.

In conclusion, our results show the importance of information concerning the conditions affecting GHb methods in a particular clinical setting. In addition to the presence of Hb variants, anaemia is a major source of negative interference. Haematological status should always be considered to ensure the correct interpretation of GHb results.

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